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Method and Device for the Separation of Biomolecules

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## Background of the Invention

### Description

The invention as presented concerns methods and devices for the single or two-dimension separation of biomolecules in gels by means of electrophoresis in an electrophoresis apparatus and serves in particular the separation of, for example, proteins, glycoproteins, lipoproteins, nucleic acids or cell complexes in gels (such as in polyacrylamide gels, urea gels or agarose gels) in two dimensions.

Proteins and peptides are biopolymers which occur in thousands in each individual cell; they are the immediate gene products which catalyse, stimulate and regulate all cell processes. The structure and functional analysis form the basis for the clarification of all significant cell activities and is the basis for the understanding of illness processes on the molecular level and in molecular medicine, e.g., in the emergence and formation of tumors and for the development of early diagnosis and new therapeutics in the pharmaceutical industry. Due to the very frequent low level concentrations in which the peptides and proteins occur in the cell, it is necessary to develop highly sensitive verification methods and separating techniques for their characterisation.

Proteins are made up from long chains of amino acids and indicate a specific spatial structure for each individual protein. Each protein has an individual amino acid sequence, the primary sequence, which unfolds into a specific spatial structure (3D-structure), which is a carrier of the physiological function of the cell activity. Proteins, as direct gene products, adopt a central position in all life processes. They catalyse the biosynthetic processes as enzymes, build up the organism as structural proteins, take care of and manage the substance transport and signal transduction and play a significant role in the translation of the genetic information (protein biosynthesis) and in all regulation processes. One single cell contains over 5000 different proteins.

Biosynthesis and expression of each individual protein are precisely regulated. A variety of expression patterns occurs in different tissues. In addition, post-translational changes can occur in the proteins, such as phosphorylation in the heat shock protein 27, which are of physiological significance and result in the two-dimension electrophoresis in a characteristic pattern after immune-chemical colouring in the western-blot. For this reason, it is important to study the expression not only on the gene and messenger nucleic acid (mRNA) – level, but also to have exact and precise knowledge about the proteins, their composition and concentration in the various cells and tissues, particularly where the human being is concerned. Following total sequencing of complete genomes, such as that from yeast or as designed in the human genome project, the research of the proteins and their cell functions is subsequently a matter of central significance. In a few years from now when the human genome comprising about 100.000 genes is known, scientists will be compelled to devote more and more attention to the research of proteins as coded by the genes; this all goes together under the heading "proteome research". Up to the present, only a fraction of these important biomolecules are known on the molecular plane. Without the structure information, however, the processes of the cell metabolism and its regulatory correlations cannot be understood. This is particularly indispensable when investigating and determining the origin of diseases. Many of the over 5000 cell proteins are known neither in their structure nor in their function. In order to understand their biological significance and their physiological role, total cell extracts or the total proteins from single cell lines are separated in modern research projects into high-resolution two-dimension gel electrophoreses (2DE) and made visible. Cell extracts from various tissue specimens, such as from tumor tissue, can be compared with one another and compared also with healthy tissue specimens; the proteins can be identified and characterised by protein-chemical and mass spectrometric analyses. In this way, disease-associated proteins can be verified which can serve as markers for early recognition of the diseases or which can precisely characterise the respective degree of the disease, such as the originating phase of tumors.

As the proteins can frequently be only expressed in small amounts, it is important to develop highly sensitive methods for separation and identification. In order to be able to accomplish the ambitious and future-orientated research projects in conjunction with the genome functional analysis, new and innovative separation and analysing techniques are urgently required.

Single and two-dimensionally performed gel electrophoreses of proteins, nucleic acids or other biomolecules have been implemented for quite some time in the biochemical and pharmaceutical industries as well as in medical research and laboratory techniques for the purpose of carrying out quick separations, comparisons of separating patterns or quality control of isolates and products. The electrophoresis chambers and their accessories (power supply, cooling systems, detectors) have long been part of the indispensable inventory of every modern laboratory in the fields of natural sciences, pharmaceuticals or laboratory-medical applications.

For the separation of biomolecules in a direction of separation (single-dimension electrophoresis, 1DE), numerous electrophoresis chamber systems and separating methods are available which are adapted to the specific applications depending on the separating problem involved. The separation of single specimens is usually carried out in capillary tubes or gel strips, and the separation of multiple specimens is usually performed in flat gels (slab gels) where deposit pockets are recessed at the upper end of a thin out-polymerised gel layer. These gel pockets are prepared in the casting process by means of the introduction of so-called combs during polymerisation. The specimens are put into these pockets after polymerisation of the gel and removal of the combs. For separations of more complex protein and peptide mixtures in long gels (>10 cm), far less chamber systems are available.

For the separation into two different dimensions (2DE), where different conditions for the separation in the two dimensions are selected (e.g., first dimension: separation according to charge, second dimension: separation according to molecule size), no uncomplicated and easy-to-handle complete systems are available which would be suitable for a fully automatic processing mode. Normally, with these electrophoreses each dimension must be carried out in another chamber, and the separating gel recovered after the first dimension must be applied manually to the prepared gel of the second dimension and polymerised in there. Only then can the electrophoretic separation of the specimen be carried out in the second dimension. The incremental steps performed manually are quite time-consuming, require considerable skill, and allow perfection and reproducibility only up to a certain degree, depending on the person who is performing the handling itself.

1DE separations of proteins and peptides are usually carried out in small chambers in 5-11 cm long polymerised polyacrylamide gels having different cross linkage and different thickness (e.g., 0.7 to 1.5 mm) and width (in so-called slab gels). For this purpose, the specimens are deposited onto the gel poured in liquid form between two glass plates after polymerisation, and separated in an electrophoresis apparatus for several hours at 500 to 2000 Volt/20 cm. After this, the gel is removed and immersed in a colour solution for the colouring of the substances; after this, decolourising in a decolourising solution for the removal of the excess of colour substance. Then, the substances are visible. By means of either photography or scanning into a computer, they can be documented in their location on the gel, e.g. for comparison with other specimens. Between thin glass plates, so-called carrier-free electrophoreses can also be performed, where the substances to be separated can be separated electrophoretically in a thin buffer film without any carrier whatsoever. For other biomolecules such as nucleic acids or high-molecular complexes, agarose gels, for example, are also applied for separation purposes.

A successful alternative to the slab gels is the separation in the first dimension in fine glass capillaries (i.d. 0.7 to 1.5 mm) where the initially liquid gel mixture is applied for polymerisation and then the substance mixture is injected in from above. Firstly, the capillaries are applied with gel in a gel casting stand, then polymerised there and filled with the specimen substance, but only after they have been brought into the electrophoreses chamber.

After the specimens have been separated under high voltage, the gels which are very soft and flexible must be pushed out very carefully out of the capillaries for placing into the colouring / decolourising solution, in which case mechanical defects can occur on the gels. Due to the difficult handling, narrow flat gels (2 x 5-10 cm thin strip gels) are also increasingly used for the 1DE. As dried ready-to-use gels, these are commercially available (obtainable, for example, from Pharmacia Biotech, Freiburg, Germany).

For the separation in the two-dimension technique (2DE), the gels from the first dimension (the gels from the capillaries or the gel strips) must be pile-bedded for the separation in the second dimension by means of manual manipulation onto a correspondingly prepared flat gel (slab gel), which is already pre-polymerised, and then polymerised onto this flat gel.

Only after this can the separation in the second dimension be carried out in the corresponding electrophoresis chamber for the 2. dimension. Following separation, the usual colouring and decolourising process is carried out with subsequent recording of the results in the documentation. Various gels and buffer systems for the performance of gel electrophoretical protein separation in two dimensions have been described: in the version first described, mixtures, for example, of ribosomal proteins are separated in urea gels where the pH-conditions in the two dimensions are varied. Processes for the separation of highly complex protein mixtures, such as those from intact cells, cell lines or tissues use in the first dimension separations for reasons involving different charging of the proteins by means of isoelectro focussing (IEF) and in the second dimension separations according to molecular size in SDS-sodium-dodecyl sulphate gels. In order to perform isoelectro focussing, either immobilones or ampholines are used which are added to the gel. With the latter technique, more than 10000 proteins of a cell lysate can be separated; the former technique with immobilones is designed for separations of about 2000 proteins. With the use of ampholine gradients, separations of more than 10000 proteins have been described (Klose and Kobalz, 1995). These high-dissolution protein electrophoresis techniques are used for the modern proteom research, the determination of the expressed proteins of a cell and their pathological changes, such as in the examination of the phase of origin of tumors. The disease-associated proteins can be identified following separation in the high-dissolution two-dimension gel electrophoresis by means of protein-chemical and mass spectrometric processes.

The US 4,666,581 describes a device for the two-dimension electrophoresis where, however, both gels are brought only in spatial vicinity. Instead of manual handling which is difficult and prone to error, a mechanism, a complicated rotary mechanism is applied to transfer the gel from the first dimension to the second dimension. The buffer vessels are not integrated. The current flow must be ensured in a complicated manner by way of filter strips.

The US 4,874,490 also describes a system for the two-dimension electrophoresis, in which case the patent does not disclose a fully integrated system and requires a gel casting frame. In addition, the buffer vessels for the cathode and anode buffer solutions are not integral components of the chamber. Furthermore, there is no cooling integrated in the system.

The separation of the first dimension is performed horizontally, and vertically in the second dimension. The gels, in the design as described, must definitely (compelling) be cast in succession in several steps.

Firstly, the second dimension must be cast in a conventional casting stand outside of the electrophoresis chamber. After casting, sealing/isolating strips must be added by way of the gel and by means of mechanical manipulation. Only after this can the gel be cast for the first dimension. Following this, the spacer is removed again. The specimen assignment in larger quantities is unsolved. Only the soaking of a membrane is described. The assignment quantity and its applied concentration is limited.

Furthermore, the DE 4244082 A1 and the US 5, 407, 546 describe a method and a device for high-dissolution two-dimension electrophoresis. The selective re-hydration is an essential factor in this case. A correlated gel ("one-molecule") is expressly used. As a basic material, only dried gels can be used and these must be re-hydrated and re-equilibrated. This is done before and after the first dimension and, separately, the gel of the second dimension is re-hydrated. No fully automatic process is possible because the gel of the first dimension is transferred into a heated equilibrating vessel and re-buffered. Given these facts, no complete and thorough integration exists.

### Summary of the Invention

For these reasons, the invention is based on the task assignment of creating methods and devices by means of which and with unsophisticated means, in one single apparatus with self-cast gels and/or dried ready-to-use gels, effective gel electrophoreses of the first and ~~second dimension can be performed manually or fully automatically. It is furthermore the task assignment of the invention to state specific ready-to-use gels.~~

This task assignment is solved according to the invention by means of the characteristics in claims 1, 9, 16, 19, 23 and 24. Purposeful embodiments of the invention are contained in the sub-claims.

A particular and distinct advantage of the invention is the fact that a full automation of the entire 2DE can be performed in an uncomplicated manner where the gels for the separation in the first dimension and the gels for the separation in the second dimension, arranged in succession or simultaneously vertical to one another, are introduced as casting gels or ready-to-use gels into an electrophoresis combination-chamber and, in the case of cast gels, are isolated and polymerised,

buffer solutions subsequently filled in, e.g., a protein extract deposited onto the gels of the first dimension and the electrophoretic separation of the first dimension at constant temperature or at fixed temperature gradient carried out with, for example, rising electric voltage, the buffer solution then suctioned off, the isolation neutralised, contact gel filled into the resulting areas between first and second dimension and polymerised out, buffer solutions filled in and the electrophoretic separation of the second dimension is performed at a precisely set temperature and constant electric power or increasing current intensity and, finally, the gels for example are developed with colouring solution and the proteins are made visible with conventional methods on the gels.

A further advantage of the invention is that the two-dimension separation in the electrophoresis chamber according to the invention is accomplished with one single device which is prepared and set up for performing both electrophoresis dimensions and the electrophoresis combination chamber has a core with cooling elements where the cooling elements on both sides of the core are arranged by means of gel chambers and buffer vessels formed from internal plates and external plates in conjunction with removable or switchable isolating elements.

- In a second embodiment of the invention it is possible that the electrophoresis is carried out in a combination chamber, however no cooling element has to be integrated in the core. An external cooling can take place by way of the buffer in the lower buffer vessel of the second dimension. A later integration of the cooling into the backward plate of the chamber is, however, also possible.

An additional advantage of the invention is that the casting of the gels takes place before depositing the substances in the new chamber system in the same space, in which also the separation of same is carried out in both dimensions of the electrophoresis. For this reason, two separate gel casting stands (one each for the first and for the second dimension) are not required and can be dispensed with. The gels for the two electrophoresis dimensions are prepared before the substance separation in the electrophoresis combination chamber and are readily available at the start of the respective electrophoresis. All manipulations for casting the gels are done in one operation before the commencement of the specimen depositing.

Advantageous is also an optimum cooling which is ensured not only in the electrophoresis chamber (for both dimensions) but also in the buffer chambers for both dimensions.

In former techniques, the gels and the buffer solutions of the first dimension are usually not cooled at all and the formerly realised cooling for the 2. dimension indicated deficiencies (temperature differences within the gels; inadequate cooling of the buffers).

Variable programming operations of the electrophoresis sequence (stage programs, gradient programs, temperature programs etc.) can be carried out.

In a further embodiment of the invention, it is possible that the cooling of the gels and buffer vessels takes place by way of the cooled buffer in the lower buffer vessel of the second dimension or by means of integrated cooling devices in the backward plate of the chamber. Buffer vessels are dispensed with for the first dimension. The gel is in direct contact with the electrodes at the right and left gel end.

The electrophoresis combination chamber can also be used for one-dimension separating gels having a variable length (e.g., 10 to 30 cm) for the separation of multiple specimens. The separation can be carried out in a reproducible manner in the combination chamber under exactly stipulated standard conditions.

In the same manner it is also possible to use the electrophoresis combination chambers for the separation of several specimens, 20 specimens for example, in a one-dimension SDS-gel, where the specimens are applied with the help of a depositing comb on the gel of the 2. dimension. A corresponding application with specimen pockets is applied where otherwise the gel for the 1. dimension is contained.

In a further embodiment of the invention it is possible that only a one-dimension separation is also effected from above to below with a large number of specimens, according to which and instead of the gel of the first dimension and the isolating element, a specimen comb is applied for the production of the 2. dimension in order to obtain pockets for the specimen deposit. After the gel has been produced, the comb is removed. Then, various specimens can be introduced into the individual comb positions and separated in a one-dimension manner.



The electrophoresis combination chamber according to the invention preferentially contains two times two gels whose number can be extended at random. In a further embodiment of the invention it is possible that, for each unit, the chamber preferentially contains only the two gels for the first and the second dimension. However, numerous chambers can be randomly developed parallel and electrophoretically.

The gel pairs for the separation in the first dimension are developed parallel electrophoretically and are initially separated from the gels for the performance of the second dimension by means of a non-conductive isolation which after this, for example, is removed mechanically from the outside without unscrewing the chamber and replaced by a conductive medium which establishes the contact to the gels for the performance of the second dimension. The gels for the first and second dimension are each developed in pairs and in succession under varying conditions without the gel of the first dimension having to be transferred mechanically to the second. The electrophoresis combination chamber has for the two dimensions an integrated cooling unit as well as integrated filling and buffer vessels. The cover headpiece of the electrophoresis combination chamber contains the electric safety connections for the connection to the power supply, the connections to the cooling aggregate and all necessary filling nozzles for casting the gels, for pouring in the electrolysis puffer and the introduction of the specimen.

The 1DE/2DE – electrophoresis combination chamber according to the invention serves, for example, the separation of complex protein mixtures for the separation of proteins from tissues, cell lines or micro-organisms which can contain more than 5000 proteins. In the electrophoresis combination chamber, analytical as well as preparative electrophoreses can be performed where, depending on the substance category, e.g. isoelectric focussing and SDS-electrophoresis or separations in agarose, urea or other separating media can be used. The chamber can be thermostatically controlled, contains all buffer vessels for the performance of the first and second dimension and a headpiece which allows the performance of the electrophoreses under high voltages, such as up to 5000 volt. The electrophoresis combination chamber serves, for example, the identification of disease-associated proteins, the development of marker proteins for the establishment of early diagnosis of diseases and illnesses and the development of new types of pharmaceuticals. Cell processes such as embryonic development, transport and signal transduction processes as well as regulation and expression patterns can be studied with the help of the electrophoresis combination chamber.

It is suitable for the dissolution of >5000 proteins and is therefore an ideal and excellent instrument for examination in the proteom research, a new field of research and development in medical and pharmaceutical basic research and for industrial applications, such as for the examination of complete cell contents and their changes or for the examination of the influences of pharmaceutical products on the cell processes.

A particular advantage of a second embodiment of the invention lies in the fact that a full automation of the entire 2DE can be performed in an uncomplicated manner where the gels for the separation in the first dimension and the gels for the separation in the second dimension are arranged horizontally to each other, meaning, above each other.

This horizontal arrangement is made possible in such a way that the isolating elements between the gels of the first dimension and the gels of the second dimension are also routed horizontally in a defined zone but at the same time, however, can be drawn out by way of deflection elements to the guiding upwards out of the combination chamber.

A further advantage of the second embodiment of the invention lies in the fact that, with the same combination chamber, the one-dimension separation can be carried out where, instead of the gel for the separation in the first dimension as well as the isolating element, a comb with specimen pockets for various specimens can be placed into the SDS-gel and this is polymerised out, the comb removed after out-polymerisation, the specimens located in the resulting recesses and, subsequently, the one-dimension electrophoresis is performed.

For this purpose, the use of the isolating element is dispensed with. Instead, a comb with specimen pockets is applied during the out-polymerisation of the SDS-gel, which is removed after out-polymerisation of the gel so that multiple ports for depositing various specimens are now contained in the gel. These can all be separated parallel to each other in the one-dimension electrophoresis. The design of the combination chamber remains unchanged and one-dimension as well as two-dimension gels can be performed in the same chamber system. Advantageous is also the fact that long routes can be carried out, such as for example 32 cm long one-dimension gels.

## Brief Description of the Drawings

The invention is to be described in greater detail on the basis of embodiments illustrated in the Figures listed below and showing the following items:

- Fig. 1 An exploded view of the essential structural components of the electrophoresis combination chamber,
- Fig. 2 The structural arrangement of the internal core,
- Fig. 3 The internal core with the arranged internal plate,
- Fig. 4 The electrophoresis combination chamber with located seals and arranged isolations,
- Fig. 5 The electrophoresis combination chamber, laterally completely mounted with clamping frame,
- Fig. 6 to 10 An arrangement showing the principle of the combination chamber in a design variant in the individual completion stages.

## Description of the Preferred Embodiments

The electrophoresis combination chamber 1 for the separation of, for example, complex protein mixtures allows the electrophoretic separation in succession in two different dimensions, meaning, under different conditions.

As shown in Fig. 1, the electrophoresis combination chamber 1 consists of several parts which are assembled together by means of a threaded union: the internal core 2 for effective cooling, connected with it on both sides the internal plates 4 and the outer plates 5, between which and on both sides there is a vacant space in each case for a flat gel (1. and 2. dimension). With the help of a seal 19, these plates 4, 5 are sealed against each other and, at the same time, the thickness of the gel is stipulated (e.g., 0.75 mm or 1.5 mm). Whereas the internal plate 4 is made from material which is good in conducting the temperature (e.g., ceramic material with specially good conductive properties, thin plastic material), the outer plate 5 is preferentially made of a transparent material (e.g. glass) in order to ensure maximum viewing capability.

The outer plates 5 are kept in position with a clamping frame 13, each screwed on both sides; brackets, normally used in such cases, are not applied here. The lower limitation of the electrophoresis combination chamber 1 is established by means of an adjusting and rotary table, upon which the electrophoresis combination chamber 1 is fixed-positioned in the middle. On the electrophoresis combination chamber 1 there is a top assembly in the form of a cover 14, into which the supply line 11 and the return line 12 for the thermostatic cooling water and inlets for the buffer vessels 8 and 21 of the first and second dimension (for the anode and cathode buffers, respectively), for filling nozzles and the inputs of the electrodes are applied. The inlets for the buffer vessels 8 and 21 initially serve as filling nozzles for pouring in the gel liquids. The internal core 2 and the clamping frames 13 are, for example, made from polymer material (such as acryl glass, plexi glass). There are windows recessed in the clamping frame 13 in the size of the gels so that the casting of the gels and the electrophoresis can be conveniently viewed from the outside.

The internal core 2 (Fig. 2) and the two internal plates 4 are cemented by the manufacturer (Fig. 3). Furthermore, the buffer vessels 8 and 21 are arranged which are partially and simultaneously filling chambers for the gels. The internal plates 4 seal off tightly the cooling labyrinth 10 (cool meander), but have ports to the buffer vessels 8 of the first dimension (buffer, 1. dim.) and buffer vessels 21 of the second dimension (buffer 2. dim.), as shown in Fig. 2 and 3. the cooling elements 3, 10 are located underneath the gels and not only cool the gels of the first (gel 1) and second dimension (gel 2), but also the buffer vessels (buffer 1. dim. and buffer 2. dim.).

In Fig. 3 (core completed cemented; does not have to be dismantled after the electrophoreses), above on the right, the filling chamber 8 for the 1. dim. gel with filling tube 17a for gel 1 and, simultaneously, the buffer chamber 8 for the first dimension (route 1. dimension) is shown again.

In addition, above on the left, the filling chamber 17b with filling tube for the 2. dim. gel and simultaneously the buffer vessels 21 for the 2. dimension (route 2. dim.) are shown. The left filling chamber 17b ends below in the middle in order to facilitate a uniform pouring of the gel liquid from below to above, where a vent opening 18 with a slanted upper limitation provides for a uniform, slow and bubble-free pouring operation.

In a special embodiment, the electrophoresis combination chamber 1 is surface-coated. This surface coating is an application of amorphous carbon layers to those parts having contact with the media gel, gel solutions and buffer solutions. The advantages of the surface coating are, as a result of low surface energy, the prevention of sticking of the media constituents (analogue to teflon) and, subsequently, the convenient removal of the gel after separation as well as the convenient cleaning of the equipment itself; the coating layer is a hard material layer, has high resistance to scratching and is thermally stable. As an alternative, these surfaces can also be silanised.

In order to carry out the electrophoresis, the following work steps are performed:

1. In the first step, a two-part seal 19 is placed onto the complete core structural arrangement (Fig. 4).
2. After this, in the second step, isolating hoses 9 (e.g. 0.75-1.5 mm round hoses or square material, such as silicone) are drawn into pre-specified recesses (Fig. 4). These serve to form the lateral boundary limit of the 1. gel from the 2. gel and the lateral boundary line of the 2. gel to the outside.
3. In the third step, the outer glass plate 5 is placed in position as required.
4. In the fourth step, the clamping frame 13 is placed in position and tightened in its location with the screws (Fig. 5).
5. In the fifth step, the body which has now formed is turned around and steps 1-4 are repeated analogously for the preparation of the parallel gels.
6. In the sixth step, the two gels are cast for the first dimension into the gel chambers 6 formed between the isolating hoses 9, after this and immediately the two gels into the gel chambers 7 for the second dimension.
7. The cover 14 is placed on its position and the polymerisation can, for example, be carried out overnight.
8. After this, the buffer solutions are filled in and then the protein extract after high-speed centrifuge can be deposited onto the 1. dim. gels.
9. This is followed by the electrophoretic separation in the first dimension.
10. After this, the 1. dimension electrophoretic puffer is suctioned off and the two isolating hoses 9, which separate the 1. gels from the 2. gels, are drawn out from the outside, something which takes place very easily, and the resulting free capillaries between the gels are filled by filling in stacking liquid, so that, after polymerisation of these liquids, the contact between the respective first and second gel is established.
11. Then the electrophoresis in the second dimension is performed.

12. After performing the electrophoresis in the second dimension, the threaded unions are undone, the gels together with the outer plates 5 removed and placed into a colouring and discolouring bath. Following this step, the gels are ready for the documentation.
13. The performance of new gels is possible again and immediately after cleaning of the gel plates and the filling and buffer vessels.

With the assembly of several units, up to 10 2DE gels can be carried out in one work operation.

When casting the gels of the first dimension, the gels as flat gels are poured in the U-tube formed by the filling tube 17a and the gel chamber 6, and there they are polymerised out. For this purpose, a stop gel with a high cross linkage is cast which is intended to fill out the lower area of the U-tube. The gel solution which is still in liquid form after the addition of a polymerisation starter is put, for this purpose, into the outer buffer reservoir of the first dimension, in which case the gel fills out only the lower area of the U-tube. It reaches about 10 mm beyond the lower end of both isolating elements formed as isolating hoses.

After the polymerisation processes has been completed, the separation gel for the first dimension is cast. In this gel, the protein separation is performed by isoelectro focussing, for example. For casting, the separation gel solution which is still in liquid form is poured into the second (located further inside) buffer reservoir of the first dimension by way of the filling chamber 8 until the area of the first dimension between the two isolating elements 9 is completely filled. The casting of the gels is done under constant temperature (e.g., at 20 °C, for which purpose the cooling is active) until the gel is polymerised out.

The gel of the second dimension is poured in between the two isolating elements 9 from below into the apparatus, where the gel solution is filled in by way of the casting reservoir of the second dimension. The gel solution flows from there via the filling tube 17b in a downward direction and emerges in the middle of gel chamber 7. As a result of the pouring operation, the air is automatically is displaced upwards and can escape through the vent opening until the gel solution has completely filled out the area of the second dimension.

The gel polymerises out at a constant temperature (e.g., by cooling to 20 °C).

The performance of the first dimension is described as follows:

The two buffer reservoirs (8) of the first dimension are filled with an alkaline and with an acidic buffer solution (e.g., 0,1N NaOH; 7% v/v H<sub>3</sub>PO<sub>4</sub>). The one solution flows here into the U-tube to the stop gel whereas the other solution coats over the separation gel. The separation gel is then coated over with a protective solution having a higher density than the buffer (e.g., 6% (w/v) glycerine, 4 M urea, 1,5% (w/v) ampholyte in H<sub>2</sub>O). The specimens can be deposited by undercoating under the protective solution onto the separation gel of the 1. dimension by way of the filling opening 20. The electrophoretic separation is then carried out e.g., at constant temperature (e.g., cooling to 18 °C) with a voltage gradient of initially e.g. 100V to e.g. 3000V over several hours. After completion of the separation, the buffer solutions are suctioned off.

For the performance of a 2DE, the specimen must always be deposited on the gel of the first dimension; the separated bands after the first dimension are then further separated in the second dimension.

Before commencing the electrophoretic separation in the second dimension, the three isolating elements 9 are removed by drawing them out to the outside. The cavity space between the gels of the first and second dimension is filled with a contact gel (e.g. agarose) which polymerises out quickly. Then, the two buffer vessels 21 of the second dimension are filled with running buffer (e.g. SDS-running buffer: 192 mM glycine, 25 mM tris-base, 5 µM bromophenol blue in H<sub>2</sub>O). The separation is performed under temperature to e.g. 15°C, e.g., at constant capacity or with a current intensity of e.g. 40 mA per gel in the first 20 minutes and then with 75 mA per gel. The separation is finished upon reaching the marker colouring substance bromophenol blue at the end of the gel. The electrophoresis combination chamber is unscrewed and dismantled and the gel removed for developing and colouring.

In accordance with the state of the art, the electrophoresis gel is then further treated and put into e.g. a trough with fixer solution. By means of silver colouring, the proteins in ng-quantities can be made visible for analytical purposes or can be treated by means of e.g. Coomassie Brilliant blue for micro-preparative purposes (e.g. subsequent enzymatic separation and sequencing).

As an alternative, the gel after blotting for immune colouring can be developed with corresponding antibodies. These steps are not an integral part of the invention but are indispensable in order to make the proteins visible after the separation has been performed.

In the embodiment as presented here, the gels used are flat gels ( no longer as round gels), but narrower than in the previous flat gel systems. The width of the gel can be variably upheld by the selection of differently wide sealing strips, hoses etc.), so that not only analytical but also micro-preparative gels can be made. In the standard commercially available flat gel electrophoresis combination chambers, ready-to-use gels with certain widths and thicknesses are used. Moreover, in the method in question, ampholines (soluble zwitter molecules) are used instead of immobilised zwitter ions (immobilones) in order to build up the pH-gradient in the gel. In principle, immobilones can also be used in the device according to the invention. However, the separating characteristics of these gels have not been as good up to the present compared with those produced with ampholines.

The advantages of the described electrophoresis combination chambers are:

- variable widths of the gels;
- longer separating sections without the danger of damage to the gels;
- application of variable protein quantities in the same apparatus;
- no transfer necessary of the gel from the first to the second dimension;
- no re-hydration necessary as in the case with ready-to-use gels;
- when using ready-to-use gels, no application of increased temperature in the way it is applied for re-hydration steps;
- ready-to-use gel strips as well as self-cast gels can be used;
- precision fixation of the gel relative to the 2. dimension;
- buffer reservoirs incorporated in the chamber system;
- cooling of both gels can be carried out on a routine basis;
- the cooling allows identical temperature profiles for both parallel gels by means of meander-shaped forced circulation of the cooling medium; no different flow conditions;
- cooling of all buffer vessels (for the 1. and 2. dimension);
- introduction of the so-called stop gel (serving the mechanical but not the electric separation of the gel of the first dimension to a related buffer vessel);



- simultaneous casting of both dimensions is possible; this saves time as the gels must polymerise before use, meaning, both gels can polymerise out at the same time (e.g. overnight) and are then ready for use in the early morning hours;
- separation is performed horizontally and not vertically so that no tilting operations are necessary. The position of the chamber does not change during the gel casting and during the electrophoresis. The apparatus is standing upright after assembly, meaning, space is saved as a result.

After carrying out the first dimension, a new feature is that the isolating hoses 9 or the isolating strips 9 can be removed without opening the chamber after the first dimension. Full automation is possible with the use of a stepper motor for removing the isolating elements 9. A new feature is also the introduction of a conductive contact liquid (e.g. on agarose basis) in the resulting cavity between the gel of the 1. and the 2. dimension, so that the electric transition to the 2. dimension is ensured without the necessity of a re-buffering. However, the re-buffering certainly take place where the resulting interim space after removal of the isolating hoses 9 is purged (flushed) with buffer solution.

In the second dimension, a new feature is that the filling of the gel solution for the second dimension is carried out from below with an upright position of the electrophoresis combination chamber. The solution is automatically filled in slowly (due to the diameter of the filling chamber) so that bubble formation is thoroughly avoided, a problem which normally occurs and has to be confronted with most chambers.

A new feature is also the vent opening 18 of the 2. dimension, bevelled upwardly, so that all of the air can escape.

This is possible because the gel in the gel chamber 7 is, however, standing vertically but the proteins are separated horizontally (from right to left or from left to right, depending on the selection of the electrode connections).

In the system, so-called ready-to-use gels, meaning, dehydrated gels in the electrophoresis combination chamber can also be used.

For this purpose, a strip-shaped gel (1. dimension) is deposited onto a carrier foil. Next to this strip, a flat gel (2. dimension) is deposited at a certain distance.

The gels on the carrier foil are placed on plate 4 above the cooling elements. After this, the outer seal 19 as well as the isolating elements 9 (one to the left and one to the right of the gel of the first dimension, in each case) are positioned.

The glass plate 5 is placed on position and the clamping frame 13 tightened as required.

Re-hydration solutions for both gels are filled into the buffer vessels 8 and 21.

The gels are re-hydrated. They swell in the same chamber in which the subsequent electrophoresis is performed. After re-hydration, excess buffer is removed and the specimen is deposited. Then, the running buffer for the 1. dimension is filled into the filling openings 8 and 20 and the electrophoresis in the first dimension is carried out. Following this, instead of the running buffer, re-equilibration buffer for re-equilibrating on the buffer conditions of the second dimension is filled into the openings 8 and 20 and removed after completed re-buffering the re-equilibration buffer is removed, and the isolating element 9 formed as a sealing hose is drawn out. The resulting space is filled with collective gel solution which polymerises out quickly.

The buffer solution for the second dimension is filled in and the second separation is started.

The use of specially developed and dried gels has the advantage that these gels can be seal-welded into foils, conveniently packed and dispatched. IEF ready-to-use gels, in which ampholines are used as zwitter ions, were developed. Worldwide, they have not yet been described and are therefore not commercially available. In the electrophoresis combination chamber, immobilised immobilone ready-to-use gels can also be used which, for example, are sold by Messrs Pharmacia. By contrast with the chambers as sold by Pharmacia, these ready-to-use gels, according to this solution as presented, can also be re-hydrated in the electrophoresis combination chamber according to the invention, whereas at Pharmacia the re-hydration is done in separate dishes so that the re-hydrated gels have to be transferred into the IEF-chamber. As an innovative feature, the invention as presented also uses combinations from both IEF gel types; e.g., ampholine ready-to-use gels whose ends are manufactured with immobilines so that the separating range on the acidic and basic separating side of the IEF gels can be considerably extended.

In order to document the separating technique in the separating chambers according to the invention, the most important recipes for separating gels under special standard conditions are stated as follows. Depending on each protein mixture, of course, special conditions have to be adapted in addition to the standard conditions.

An alternative design variant is the application of thin-walled, high-elastic hoses as isolating elements 9. The separation of the vacant space for accommodating the gel of the first dimension is realised either by a hose which is put in U-shaped (possibly in an envisaged groove in one of the plates) or by two hoses, each of which is closed off at one end. The sealing effect is produced by filling the hose with a fluid (liquid or gas). With sufficient internal pressure, the sealing against the inner and outer plates 4 and 5 is effected. After casting the stop gel, the gel of the first dimension can be cast. The limitation of the gel of the second dimension is done analogously with a U-shaped hose or with a hose closed off at one end. After separation of the first dimension, the hose is evacuated but not removed from the system. An agarose solution is filled into the resulting space between the first and the second dimension for the purpose of establishing an electrically conductive connection between the gels. The other processes are performed as described above.

An advantageous formation exists in the fact that this hose is fixed-positioned with an adhesive substance only on one of the plates 4 or 5, or solidly seated on one of the plates 4 or 5 in a groove, so that it is ensured that the hose after evacuation is located on one plate 4 or 5.

A variant of this design or method, respectively, is to remove the hose after completed separation of the first dimension. By means of the evacuation, the removal is substantially simplified.

The combination chamber according to the second embodiment consists of two parts which are arranged above each other, the upper IEF-part for the performance of the IEF-electrophoresis in the first dimension and the lower part for the performance of the SDS-electrophoresis in the second dimension.

The combination chamber consists of several components which are, for example, assembled together either by means of a threaded union or clamping. The rear wall plate 28A forms one unit with the upper buffer reservoir 29 of the second dimension and the casting vessels 30 for casting the second dimension, the buffer vessel 31 for filling in the buffer of the second dimension. The cover plate 28B designed as a glass plate is connected with the rear wall plate 28A and the upper buffer reservoir 29 by means of, for example, a threaded union. Flat seals 23, right and left, seal off to the outside and set the thickness of the gels between the plates 28A and 28B. The plate 28B has a height which results from the height of the plate 28A and the height of the upper buffer reservoir 29. The plates 28A, 28B can be transparent. In particular, the upper cover plate 28B should be transparent in order to observe the individual steps and stages of the process.

Between the deflection elements 22, in the embodiment as presented here, there is an isolating element 24 in the form of a U-shaped hose seal which separates the gel 25 of the first dimension from the gel 36 of the second dimension. The electrodes 26 and 27 for the electrophoresis of the first dimension are located next to the deflection elements 22.

The assembled construction consisting of the plates 28A, 28B and the upper reservoirs is placed in the lower buffer tank 32 of the second dimension. At this location there is a seal 33 which, for example, can be lifted pneumatically (status 33a) in order to seal off in the downward direction the empty space between the plates 28A and 28B, so that the gel 36 of the second dimension can be cast by way of a connection (e.g., a hose) from the casting vessel 30. The buffer filling vessel 31 serves the purpose of filling the lower buffer tank 32. The electrodes 38 and 39 of the second dimension are located in the buffer vessel 29 and the buffer tanks 32.

The assembly of the combination chamber is described as follows:

For the preparation of the 2DE, gaskets 23 as spacers made of plastic material are placed right and left onto a glass plate 28B, from which two deflection elements 22, e.g., bolts, are protruding (refer to Fig. 6). The areas of the first and second dimension are merely separated from each other by means of an isolating element 24, in this case an expandable plastic hose, which is arranged in a U-shaped manner between the two deflection elements 22 in such a way that a straight separation in a downward direction results. It protrudes at both ends of the upper chamber and can be drawn out easily.

Between the deflection elements 22, in the embodiment as presented, an IEF ready-to-use gel strip 25 is positioned (white horizontal surface) and brought into direct contact with the two electrodes 26, 27 which protrude through the glass plate 28B at the deflection elements 22, allowing the electrophoresis in the first dimension. Then, the upper glass plate 28B is placed on as required (Fig. 7) and, in this embodiment as presented, is held together by means of clamps with the lower rear wall plate 28A and the upper buffer reservoir 29. The glass plate sandwich is then placed in the buffer tank 32 (Fig. 8). The pouring vessel 30 serves the purpose of casting the SDS-PAGE-gel, and the reservoir 29 and the buffer tank 32 as buffer reservoir for the SDS-page, where the buffer tank 32 is filled from buffer filling vessel 31 by way of a connecting line.

In the buffer tank 32 there is a seal 33 which, when sealing off, can close the slot between the two glass plates 28A, 28B on the lower side. It is shown in a non-sealing state in Fig. 8.

In the embodiment presented here, the preparation and performance of the electrophoresis is carried out as follows:

The IEF-gel 25, for re-hydration with re-hydration buffer between the glass plates 28A, 28B, is coated over in area 34 and re-hydrated. After re-hydration (>2h) the excess buffer is removed. Then the specimen is put into a recess 35, whose place during the re-hydration remained recessed by means of the application of a spacer. After this, the electrophoresis is carried out by applying a voltage between the electrodes 26, 27. After the IEF-electrophoresis, the IEF-gel 25 is rebuffed (e.g. 30 min, at pH 6, 9) by adding re-equilibration buffer which is applied in area 34.

The SDS-gel 36 for the second dimension is cast before, after or during the performance of the first dimension, where the gel solution is filled in by way of the pouring vessel 30 and conducted downwards between the glass plates 28A, 28B (Fig. 9). In this condition, the seal 33A seals off the glass plate sandwich in the downward direction, so that the gel solution cannot run out. The gel 36 is polymerised for a minimum of two hours between the glass plates 28A, 28B. After this, the sealing is again neutralised by the seal 33A, so that the SDS-gel enters contact with the electrophoresis buffer in the buffer tank 32.

After completion of the IEF-electrophoresis and after rebuffering of the gel strip 25, the re-equilibration buffer is removed from area 34, the plastic hose 24 is drawn out from the apparatus laterally and in the upward direction, with e.g. a stepping motor, and the resulting interim space 37 between the first and second dimension is filled with contact gel (e.g., agarose collective gel (Fig. 5). Then, buffer is filled into the buffer reservoir 29 and the buffer tank 32. By way of an electric field between the electrodes 38, 39 of the second dimension, in the buffer vessels 29, 32 filled with buffer the SDS electrophoresis in the second dimension is performed. Following completion of the electrophoresis, the plate sandwich is taken out, the gel lifted off and developed according to known methods, e.g. colouring with silver nitrate solution or coomassie solution.

The cooling of both electrophoresis dimensions is done by immersing the gel sandwiches into a thermostatically controlled buffer solution of the second dimension which is in the buffer tank 32, or the chamber sandwiches are cooled by the cooling chambers placed against the glass plates 28A, 28B.

New IEF-gels (isoelectric focussing gels) according to the claims 24 to 29 are also subject-matter of the invention. They offer an excellent separating effect for biomolecules, particularly for proteins, and an extended separating range on the acidic and basic separating side. As follows, the selected recipes of gels are documented.

### **Standard recipes**

#### **Ampholine ready-to-use gels (hydrated gel)**

3.5 – 4% acrylamide gel with 9 M urea with minimum 2% ampholines (WITA ampholyte),  
pH-range pH 2.0 to 11.0  
(with or without additive of detergents and thio urea)

#### **Ampholine-immobilon ready-to-use gels (hydrated gel)**

3.5 – 4% acrylamide gel with 9 M urea with minimum 2% ampholines (WITA ampholyte),  
pH-range pH 2.0 to 11.0  
(with or without additive of detergents and thio urea)

lateral immobiline gels: 10% acryamide gel with additive of 50 mM to 100 mM immobilines.

SDS-gel, 2. dimension:

SDS-PAGE with 15% acrylamide, 0.1% SDS, Tris/HCl-buffer, pH 8,8

Re-hydration buffer

9M urea, 2-4% ampholine, pH range 2-11

(with or without additive of detergents and thio urea)

Re-equilibration buffer

3% SDS, 70 mM DTT, Tris/phosphate, pH 6,8

Agarose collective gel

0.1% SDS, 1% agarose, Tris/phosphate, pH 6,8

Electrophoresis-buffer 1. dimension

Buffer A: 4% (v/v) phosphoric acid

Buffer B: 5% (v/v) ethylene diamine

Electrophoresis-buffer 2. dimension

SDS-running buffer: 192mM glycine, 25 mM tris-base, 0.1% SDS

### Run conditions:

#### 1. Dimension:

1h	100V
1 h	200 V
17.5 h	400 V
1 h	600 V
0.5 h	800 V
10 min	1500 V
5 min	2000 V

#### 2. Dimension

30 min	40 mM
6 h	80 mM

The invention is not restricted to the embodiments as listed here. Moreover it is possible, by means of combination and modification of the means, utilities and characteristics as stated herein, to realise further embodiment variants without departing from the outlining framework and scope of the invention itself.



## Reference Numbers List

- |   |   |
|---|---|
| <p>1 Electrophoresis combination chamber</p> <p>2 Core</p> <p>3 Cooling elements</p> <p>4 Inner plate</p> <p>5 Outer plate</p> <p>6 Gel chamber</p> <p>7 Gel chamber</p> <p>8 Buffer vessels and filling chamber for the 1. dimension incl. inlets</p> <p>9 Isolating elements</p> <p>10 Cooling labyrinth</p> <p>11 Supply cooling liquid</p> <p>12 Return cooling liquid</p> <p>13 Clamping frame</p> <p>14 Cover</p> | <p>15 Clamping elements</p> <p>16 Viewing window</p> <p>17a Filling chambers for the 1. dimension</p> <p>17b Filling chamber with inlet for the 2. dimension</p> <p>18 Venting openings</p> <p>19 Surface seal</p> <p>20 Fill-in opening for specimen and 1. dim. gel</p> <p>21 Buffer vessels incl. inlets for 2. dimension</p> <p>22 Deflection element</p> <p>23 Seal</p> <p>24 Isolating elements</p> <p>25 Gel of the first dimension</p> <p>26 Electrode of the first dimension</p> |
|---|---|

- |     |  |    |  |
|-----|--|----|--|
| 27  | Electrode of the first dimension   | 35 | Recess in the gel of the first dimension for specimen deposit                    |
| 28A | Rear wall plate  | 36 | Gel of the second dimension  |
| 28B | Cover plate  | 37 | Interim space between plates (28A, 28B), filled with contact gel                 |
| 29  | Upper buffer reservoir of the second dimension   |    |  |
| 30  | Pouring vessel of the second dimension   | 38 | Electrode in the upper buffer vessel for electrophoresis of the second dimension |
| 31  | Buffer filling vessel of the second dimension  | 39 | Electrode in the lower buffer vessel for electrophoresis of the second dimension |
| 32  | Lower buffer tank of the second dimension  |    |  |
| 33  | Seal (can, e.g., be pneumatically lifted   |    |  |
| 33A | Seal (is, e.g., pneumatically lifted)  |    |  |
| 34  | Area between plates (28A, 28B) above the hose seal (24), which is filled with buffer for the re-hydration and re-buffering of the gel of the first dimension |    |  |